How T cells talk to the neighborhood: the spatiotemporal dynamics of T cell-derived cytokines in cancer

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5

A chapter of negative data

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Undoubtedly, all scientists will have experienced that 'negative results', i.e. data that are inconclusive or fail to reveal a clear trend or effect, constitute a (large) part of their experimental output. At present, the majority of this negative, but potentially valuable, scientific information remains unshared with the wider community, as scientific publishing is heavily biased towards the reporting of successful experiments [1]. This phenomenon not only skews the scientific literature, but also leads to the needless repetition of time and money-consuming experiments by others, wasting resources and in some cases perhaps also scientific enthusiasm. Given that actual publication costs have become less of an issue in the present time of digital data sharing, it would be valuable if negative results would be made accessible to the scientific community on a regular basis. In this chapter, we would like to provide an example by informing the field on a yet not understood difficulty in the generation of an IFNγ sensing reporter mouse.

Generation of a (non-) IFNγ-sensing reporter mouse

T lymphocytes are important mediators of adaptive immunity against pathogens and cancerous cells. Upon activation, antigen specific T cells rapidly expand, acquire effector functions, and travel to the site of infection. There, they secrete cytotoxic effector molecules that can directly kill the target cell, assuring quick clearance of the diseased cells. In addition, T cells are able to release cytokines after stimulation through their antigen receptors, as a "cry for help" towards other cells, to control the infection [2]. One of the key T cell-secreted cytokines is IFNγ, an effector molecule with pleiotropic effects during an immune response. For instance, signaling through the IFNγ receptor (IFNγR) can induce the expression of the CXCL9, 10 and 11 chemokines that attract activated T cells to the target site[3]. In addition, IFNγR signaling enhances the expression of components of the antigen presentation machinery [3] and can have direct cytopathic and cytostatic effects on both cancer cells and virally infected cells [4-6]. Finally, the expression of immune checkpoint molecules such as PD-L1 and PD-L2, which are important in dampening immune activity during chronic infection but can also suppress tumor-specific T cell responses, are under control of IFNγR signaling [7].

Despite the very well-documented role of IFNγ in the control of infection and cancer, little is known with respect to the spatiotemporal aspects of IFNγ sensing: How far do T cell secreted signals reach in the surrounding tissue? Is such spreading substantially influenced by the extracellular matrix composition of the tissue microenvironment?

And are there amplification loops in which cells that are close the secreting T cell, start secreting signals themselves, thereby increasing the spread of the "cry for help"? In previous studies, whole transcriptome and immunohistochemical analysis of virus or parasite infected skin have been used to demonstrate that triggering of T cells within the skin can lead to the IFNγ mediated expression of a broad spectrum of general host-defense related genes in a large fraction of surrounding skin cells [8, 9]. Related work on IFNγ sensing in lymph nodes has demonstrated that secreted IFNγ can induce expression of IFNγ responsive genes in cells outside infected areas [10]. Using mouse transplantable tumor models in which tumor cells were modified to express a genetically encoded IFNγ sensing reporter, it was recently demonstrated that IFNγ secreted by activated $CD8^+$ T cells leads to IFNγ sensing in antigen negative 'bystander' tumor cells, with such sensing being observed hundreds of micrometers away from the site of antigen-T cell interaction [6, 11]. Together, these data provide the first visualization of the effects of T cell-secreted IFNγ on surrounding cells within a micro-environment. At present, questions remain about the activity of IFNγ on an even larger scale, i.e. that of the complete animal: To which extent does IFNγ stay local or causes a systemic response? What are the cell types that respond and where are they localized? What are the kinetics of such a response and do immune therapies influence these parameters? To address these questions, here we set out to generate an IFNγ-receptor signaling reporter mouse in which cells will "light up" after IFNγR signaling. This system should allow us to determine which cells in the mouse's body respond to IFNγ and how this response manifests itself through space and time.

For the generation of transgenic IFNγ sensing reporter (IGS) animals, we cloned an IFNγR-signaling responsive promoter containing IFNγ-activated sequences (GAS), that are found in the promoter sequences of virtually all genes that are upregulated by IFNγ [12], followed by a red fluorochrome (Katushka) and the tamoxifen inducible Cre^{ERT2}. In this way, we aimed to both follow the direction and velocity of IFNγ in real time (using the transient expression of Katushka as a marker), as well as the entire size of the cell field that a single (T) cell can cover when activated (using Cre^{ERT2}, providing us with a permanent mark after crossing the inducible reporter mouse to, for instance, a lox-stop-lox-GFP mouse) (figure 1a). We tested several variants of the GAS-constructs *in vitro* using lentiviral transduction, and two different designs, one containing a 4xGAS-minimal CMV-promoter ((GAS: 5′-agtttcatattactctaaatc-3' ;mCMV: 3'-cggtaggcgtgtacggtgggaggtctatataagcagagctctctggctaacta-5') GASm) and a second containing a 4xGAS-tatabox-promoter (GAS: 5′-agtttcatattactctaaatc-3' ;Tatabox: 5'-tatataagcagagctctctggctaacta- 3′) GASt) were used for the generation of two versions

of the reporter mouse. In brief, separate *in vitro* transduction experiments demonstrated that IFNγ stimulation induced Katushka expression by cells expressing each of the two vectors. Both constructs induced comparable katushka mean fluorescent intensities, and a clear signal to noise ratio could be observed in transduced cells, including cells carrying a single copy of the (GASt) transgene (figure 1b-d). Furthermore, Katushka expression was observed following exposure to concentrations of 0.01 – 1000 ng/ml IFNγ (figure 1e) and in both human and murine cell lines (figure 1b-f).

Subsequently, two separate approaches were used for the generation of the IFNγ sensing reporter mouse: through the introduction of (a single) reporter transgene in the Col1A locus and via the insertion of multiple transgenes using transposon mediated transgenesis. For the first approach, C57BL/6 derived embryonal stem (ES) cells were modified by targeting the GASm reporter construct in the Col1A1 locus via Recombinase-Mediated Cassette Exchange [13]. Subsequently, modified ES cells from two donor mice were injected in blastocysts of C57BL/6 foster mothers, yielding four chimeric animals. Germline transmission of the transgene was observed for one male mouse, resulting in heterozygous progeny, as assessed by PCR genotyping. To test whether cells of these mice showed reporter activation upon IFNγ exposure, cells from, amongst others, blood, liver, skin, spleen and testis were treated with recombinant IFNγ *ex vivo*. In all cases, no evidence for Katushka expression was observed using flow cytometric analysis (figure 2a). Also when ES cells were directly tested for IFNγ responsiveness, no Katushka signal was observed (figure 2b). With our second strategy, we obtained mice carrying 7-9 copies of the reporter in their genome (measured by qPCR analysis), as a result of zygote injections with transposase mRNA and a pT2BH sleeping beauty vector including our GASt transgene. To determine whether IFNγ sensing could be observed in this mouse model, a cohort of these mice was infected intravenously with *Listeria monocytogenes* (eliciting a large T cell mediated IFNγ response) and blood, and the main infected organs liver and spleen were analyzed for Katushka expression at two timepoints (day 4 and day 8) during the T cell response. Also in this mouse model no reporter positive cells were observed (figure 2c).

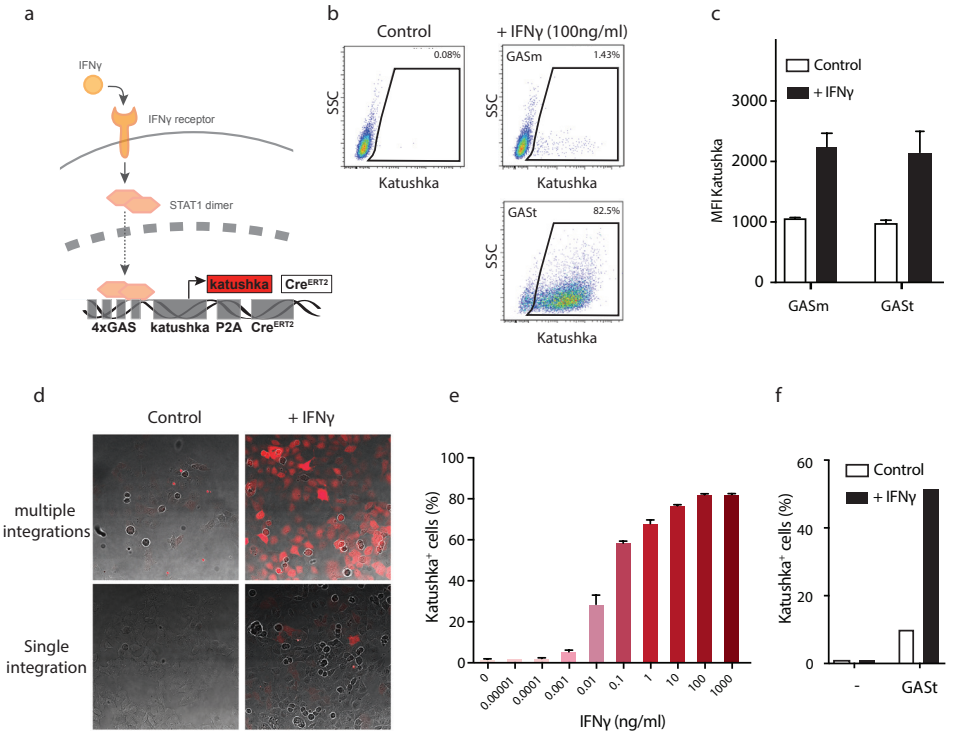

Figure 1. In vitro characterization of the IFNγR signaling (IGS) reporter system in human and murine cells.

a) Schematic representation of IGS reporter system

b) Representative flow cytometry plots used to identify Katushka$^+$ cells within the IGS reporter-modified Hela cell population upon 24h incubation with control or 100 ng/ml recombinant IFNγ. Generally, after transduction with equal amounts of viral reporter DNA, a substantial higher fraction of cells was modified with the GASt transgene then with the GASm transgene.

c) Median Katushka fluorescence intensity of IGS reporter-modified Hela cells upon 24h incubation with 100 ng/ml recombinant IFNγ. Bar graph shows mean of n=3 technical replicates.

d) Representative confocal images of Hela cells, lentivirally transduced with the IGS reporter vector (GASt), with or without addition of recombinant IFNγ (100 ng/ml for 24h). Cells in the lower panels were treated with a low viral titer, at which the majority of the cells is likely to incorporate a single copy of the reporter.

e) Percentage Katushka-expressing cells of GASt-IGS reporter-modified Hela cells upon incubation with the indicated concentrations of recombinant IFNγ. Bar graph shows mean of n=2 technical replicates.

f) Percentage Katushka-expressing cells of GASt-IGS reporter-modified Mouse Embryonic Fibroblast (MEF) cells upon 24h incubation in the presence or absence of recombinant IFNγ (100ng/ml).

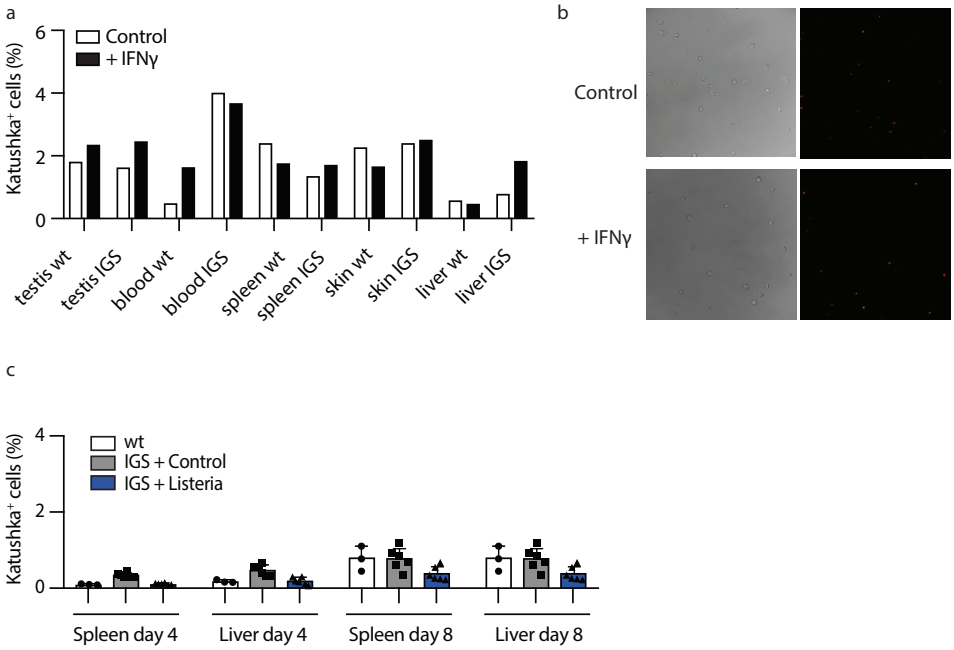

Figure 2. Characterization of Col1A1 targeted- and transposon based- IGS reporter mice.

a) Katushka-expression by cells derived from indicated organs of a wild type (wt) mouse or a second generation (F2) IGS reporter mouse that carries an integration of the GASm IGS reporter transgene in the Col1A1 locus. Bar graph shows the percentage of Katushka positive cells upon ex-vivo incubation with 100 ng/ml recombinant IFNγ for 24h. Note that the lack of difference between stimulated and unstimulated conditions is not a result of excessive background but rather of the absence of signal.

b) Confocal images of ES cells used to generate Col1A1 IGS reporter mice upon ex-vivo IFNγ exposure (100 ng/ml for 24h). Left panels: brightfield images. Right panels: fluorescent images (561nm to observe Katushka signal). Images are representative of n=3 independent ES clones. per group. Note that observed red signal is not co-localized with the cells in the left panels and should hence be considered noise.

c) Flow cytometric analysis of Katushka-expressing cells derived from the indicated organs of wild type (wt) or transposon based IGS reporter mice carrying 7-9 IGS reporter integrations, at day 4 or day 8 after intravenous injection of either PBS (control) or Listeria monocytogenes. Bar graph shows mean of n=3 (wt) or n=6 (IGS) biological replicates per group.

In summary, in this study we aimed to develop an IFNγ sensing reporter mouse. To achieve this, we generated two IFNγR-signaling responsive construct containing a series of Gamma interferon Activation Sites to control the expression of reporter proteins. These transgenes were targeted to the mouse genome using two independent approaches -either by site specific targeting to the Col1A1 locus in ES cells or by transposon-based genome targeting in zygotes. While mice carrying the correct transgene were recovered from both approaches (with 1-9 transgenes integrated per cell), we conclude that we

are not able to observe reporter expression in cells derived from these mice, either after *ex-vivo* treatment with high concentrations of recombinant IFNγ, or after bacterial infection of these mice (an approach previously shown to induce the production of high levels IFNγ by T cells and NK cells in infected organs [14, 15]. In contrast, cell lines that were lentivirally modified with the same transgenes *in vitro* showed a pronounced reporter response to IFNγ treatment.

Why do these mice fail to report on IFNγ sensing? A first, more trivial explanation would be an unforeseen technical issue such as a deleterious mutation in the transgenes. However, based on the notion that the same vectors that were used for transgenesis function well in (mouse) cells *in vitro*, and the fact that we obtain the same result using two different transgenes and separate insertion approaches, we consider this explanation less likely. A second possible explanation may be (epigenetic) silencing of the vector. Since separate gene-targeting approaches both lead to a lack of functional reporter activity, it seems unlikely that the transgene integration sites play a direct role in silencing of the reporter construct. However, it is possible that specific elements in the transgene itself would be involved. Conceivably, the repetitive nature of the four GAS elements in the promoter of the reporter construct may contribute, as repeated DNA sequences have been reported to be more prone to silencing [16], although transgenic reporter mice carrying similar constructs, for instance a Stat5 reporter containing four Stat5 response elements [17] or an NFAT reporter under the control of four ARRE2 repeating elements [18], performed according to expectations. In future attempts, a re-design of the reporter by, for example, decreasing the repetitiveness of the promoter elements or flanking the transgene with so called "insulator sequences" that help escape silencing [19] may be considered. Alternatively, integration of a fluorescent reporter directly under control of an endogenous IFNγ responding promotor, e.g. the promotor of a broadly expressed IFIT or GBP protein, may be an option to successfully report on IFNγ sensing. Finally, the use of (pre-determined) IFNγ induced gene expression signatures as a readout may be a way to identify IFNγ sensing cells (a method described in chapter 4 of this thesis), and could potentially be combined with spatial transcriptomics techniques (see chapter 7) to include information on various factors that may regulate cytokine spreading and sensing, such as for instance the presence of extracellular matrix proteins.

Considerations for sharing negative results

By sharing our attempts to generate an IFNγ sensing reporter mouse, we wish to inform other researchers who aim to explore similar avenues, thereby hopefully guiding their efforts in the most fruitful direction. To accelerate scientific progress and in the most time and cost-efficient manner, sharing of negative data or not (yet) well understood phenomena should be encouraged. What parameters do we consider important when considering to share negative data? First, the data should be sufficiently robust to ensure that the chance that the negative results are due to a trivial 'glitch' are small. Yet, for the types of projects that aim to develop new technologies, conceivably it would be too much of a burden for the researcher to try to discover ad nauseam what could be the reason for the lack of functional output, and therefore, it is all the more important that when unsuccessful attempts are shared, it is clearly communicated where possible issues may lie. Secondly, the amount of time and resources invested before being able to reach this positive or negative data point should be considered, as risk assessments should be based both on the likelihood of failure and the time/ resource investment in a project. Finally, there is an ethical component to be considered, especially when it comes to studies using animals. In particular, a moral obligation to avoid the needless use of animals provides a compelling argument to share negative results. With these rough "guidelines" in mind we would like to argue that the publication of negative or inconclusive results should be encouraged. In case of "negative" biological data, that may not support an initial hypothesis, but still describe a biological phenomenon, this should ideally be achieved by publishing the data in peer-reviewed scientific journals and/or platforms that are specifically launched to facilitate such data, as for instance the "Journal of Negative Results in Biomedicine" or "Journal of Articles in Support of the Null Hypothesis". In the field of biological engineering, this may be more challenging, as negative technical data may often be valued as failure rather than biological reality. The initiation of novel journals or journal sections covering these, such as for example "the journal for failed bioengineering projects" may reduce this issue, and may aid to a more frequent sharing of technical difficulties. Alternatively, when time is an issue, other more accessible means such as a presentation at an academic conference or "a chapter of negative data" in one's PhD thesis, will make the community aware of the scientific efforts and the chance to contact the investigators for more information. As a final note, at present, there is only little incentive for researchers to share their negative data, and the scientific field should debate how this may best be stimulated.

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